[19]中华人民共和国专利局

[11]授权公告号 CN 1022331C

[12]发明专利说明书

[21]专利号 ZL 89106580

[51]Int.Cl[5] C12N 7/08

[45]授权公告日 1993年10月6日

[24]颁证日 93.8.8
[21]申请号 89106580.6
[22]申请日 89.8.17

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A61K 39/29

说明书页数: 附图页数:

[54]发明名称 甲型肝炎减毒活疫苗毒种及其制造方法

[57]摘要

本发明是一种含有病毒性抗原的医用生物制品，特别是用于预防人类甲型肝炎的减毒活疫苗毒种的制造方法，是从甲肝病人粪便中用新生猴肾单层细胞中分离甲肝病毒，经体外连续传代，并用低温32℃减毒的方法获得甲肝减毒株，再经人胚肺二倍体细胞连续培养传代，得H2毒种，用该毒种制得的甲肝减毒活疫苗，已经三期临床人体接种观察，证明接种者无任何不良反应，可获得99%以上的保护作用，为大面积预防甲肝提供安全有效的活疫苗毒种及其制造方法。

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权利要求书

1.一种利用组织培养细胞分离、传代获得甲型肝炎减毒活疫苗毒种的制造方法，其特征是分离用的细胞为新生猴肾单层细胞，连续传15代，并置32℃低温培养，继续传5代，得HAVH2M20病毒，病毒在32℃适应KMB17细胞及继续减毒传5代，得保藏登记为CCTCC №.为92001的H2M20K5毒种。

2.根据权利要求1所述的方法，其特征是这一毒种是用一只新生猴的肾单层细胞自甲肝病人粪便中直接分离HAV并用同一只新生猴的肾细胞连续传代和筛选得到。

3.根据权利要求1或2所述的方法，其特征是制造该活疫苗的毒种是在低温32℃减毒和适应于人胚肺二倍体细胞KMB17而获得的。

4.根据权利要求1所述的方法，其特征是用HAV易感的猕猴作为检测免疫原性和安全性的动物模型。

本发明是和种含有病毒抗原的医用生物制品，特别是用于预防人类甲型肝炎减毒活疫苗毒种及其制造方法。

甲型肝炎是世界性传染病，它是由甲型肝炎病毒（HAV）引起的，全世界约40亿人受其威胁。在中国，35岁以上成年人中，甲肝受染率达90%以上，常引起季节性的流行，1988年春季的上海甲肝特大流行，患病数达约30万人以上，我国每年甲肝患病数在百万人以上，由于该病的高发病率及较长的病程，给人民健康及经济发展都有严重影响。根据我国现实情况，控制甲肝流行的有效和根本的措施在于发展疫苗，而疫苗的关键是其毒种。

美国MERCK公司Provost等人于1986年曾报告获得甲肝病毒F减毒株，但人试结果证明，该毒株的免疫效果很差，故至今未有进一步扩大人试的工作。

本发明的目的是研制出一种安全、有效的甲型肝炎减毒活疫苗毒种，用本毒种制造的甲肝疫苗经大量人群接种证明确可用于预防该病的传染，为控制该病流行提供最有效的措施。

本发明的甲型肝炎减毒活疫苗H2减毒株毒种

是用细胞直接从甲肝病人粪便中分离病毒，用组织培养传代及低温（32℃）传代法进行减毒建立对HAV易感的猕猴动物模型，并用该模型进行减毒株的筛选工作，从而获得甲型肝炎H2减毒株，经猴体试验及人体接种观察证明有良好的安全性和免疫性，是目前国际上有最多人体接种观察结果，证明对人安全和有保护效果、有实用价值的甲肝活疫苗毒种。

采甲肝病人粪便用PBS制成20%悬液，以F113—PBG沉淀及差速离心进行抽提，抽提物经超速离心沉淀，经免疫电镜检测观察到27nm大小的甲肝病毒颗粒。本分离用的细胞为来自一只新生猴的肾单层细胞。以上述初步纯化的粪便提取物接种原代猴肾单层细胞4瓶，35℃～36℃吸附2小时，用含新生牛血清的Eagles及乳蛋白维持液，置35℃～36℃培养，每周换维持液一次，至第6周，去原维持液，用胰蛋白—EDTA液消化，收集细胞，用冻融、超声波处理抽提HAV。以上述方法在该新生猴肾细胞中连续传15代，每代用直接免疫荧光监视病毒的增殖，以后以32℃～34℃培养，继续连续传5代，得HAV H2M20减毒株。

毒种适应KMB17细胞及继续减毒传代，KMB17细胞系人胚肺二倍体细胞，将上述H2M20病毒接种于KMB17（28代），置32℃～34℃培养，每28天传一代，连续传5代，得H2M20K50毒种（简称H2减毒株），该减毒株已保藏武汉中国典型培养物保藏中心（CCTCC），其保藏号为CCTCC №.为92001。

H2减毒株是通过人胚肺二倍体细胞32℃传代和用猕猴动物试验进行选育而获得的。与原来的强毒株相比，H2减毒株接种猕猴动物后，未发现病情转氨酶升高和肝细胞病变。粪便中不能直接查到甲肝抗原，但仍能诱导抗体产生。不同接种途径试验结果表明该株已失去了口服感染猕猴的能力，以上试验结果表明该株病毒对猕猴动物的毒力已经显著减弱。H2减毒株的遗传性较为稳定，回到36℃传5代未发现返祖现象。

以H2M20K5为毒种在KMB17细胞上连续传2代，制得甲肝减毒活疫苗，经人体接种观察，139名抗体阴性的人接种疫苗后连续观察6周，未发现任何与肝炎有关的症状或体征，接种后8周内

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血清谷丙转氨酶及乳酸脱氢酶第5带活性均在正常范围之内，无一例异常升高，表明该疫苗是安全的。首批接种者中4名于接种后8—30天多次收集粪便标本细后直接检测甲肝抗原均为阴性，但从其中3名用细胞培养法分离到甲肝病毒，接种者原在单位在12周内未发现有急性肝炎病例。以上情况表明接种疫苗后排病毒量很少，并未发现通过人间密切接触引起发病。

原来抗体阴性的人一次接种后用酶联免疫吸附法检测，一般于3周左右抗体阳转。阳转率达95%，抗体滴度在1：1—1：64之间，首批10名接种者于二年后复查抗体仍为阳性，对4人进行了代表保护力的中和抗体测定，证明为中和抗体。流行病学调查结果证明疫苗接种后有100%保护作用。以上结果表明疫苗株有良好的免疫原性。

用上述H2M20K5减毒株制成的甲型肝炎试验性减毒活疫苗，至今已完成了三期临床共207名健康人的接种观察，得到满意的效果。

迄今为止，国内外仅有美国MERCK公司的Provost博士等于1986年发表了他们选育出的甲肝CR326—F'减毒株人体接种的结果，共接种11人，结果仅6人有抗体阳转反应，而且开始阳转时间长达接种后7.5周。由于该减毒株的免疫效果不佳，未有进一步的报告，本发明的H2减毒株制造的疫苗，通过迄今有最多接种人数的观察结果显示，它在安全性方面进行了最详细的观察，并取得显满意的结果，证明了它是迄今最安全的活疫苗毒种，另外在免疫反应方面，抗体阳转率达95%以上，远远高于F'减毒株，特别是它首先证明本疫苗有流行病学保护效果，这些都证明，本疫苗是目前国际上有人体接种观察结果的最佳的甲肝疫苗毒种。

实施例：

采甲肝暴发点患病者粪便，原于黄疸出现前一周，该患者甲肝IgM试剂检测阳性，以往身体健康，无慢性及其它传染病，甲肝恢后康复良好，粪便用PBS制成20%悬液，以F113—PEG沉淀及超速离心进行抽提，抽提物经超速离心沉淀，以50倍浓缩于PBS中，经免疫电镜检测观察到含27nm大小的，只能与甲肝恢复期血清凝集的球形病毒颗粒。分离用的细胞为新生猴肾单层细胞。该

新生猴乃捕自武夷山的怀孕恒河猴，产于于笼内，产后立即以新生猴肾用胰蛋白酶制成单层细胞，并再消化冻存于液氮中备用。以上述初步纯化的粪便提取物接种原代猴肾单层细胞4瓶（35CM²/瓶），36℃吸附2小时，用含2%新生牛血清的Eagles及0.5%乳蛋白维持液，置35℃培养，每周换维持液一次，至第6周，去原维持液，用胰蛋白—EDTA液消化，收集细胞，用冻融、超声波处理抽提HAV，以上述方法在该新生猴肾细胞中连续传15代，每代用直接免疫荧光监测病毒的增殖，以后以32℃培养，继续连续传5代，得HAVH2M20（32℃）减毒株。

毒种适应KMB17细胞及继续减毒传代，KMB17细胞系人胚肺二倍体细胞，将上述H2M20（32℃）病毒接种于KMB17（28代），置32℃培养，每28天传一代，连续传5代，得H2M20K5毒种。

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Abstract

The present invention is related to biological products comprising viral antigen in particularly to attenuated live hepatitis A vaccine and its preparation. The hepatitis A virus strain H_2 can be obtained by separating from feces of a hepatitis A patient, and adapting to passage in vivo in newborn rhesus monkey kidney culture cell monolayer, and attenuating at a lower temperature of 32℃ in human fetal lung fibroblast cells. Clinical trials show that more than 99% of volunteers developed detectable anti-HAV neutralizing antibody, and no local or systemic complaints were present immediately after immunization or during long-term follow-up.

ATTENUATED HEPATITIS A LIVE VACCINE STRAIN AND ITS PREPARATION

BACGROUND OF THE INVENTION

Provost et al. from Merck company (J. Med. Virol., 20:165-175 ,1986) described the F and F' variants of the CR326 hepatitis A virus strain. While it is reported to be immunogenic in 11 volunteer vaccinees, the F variant also caused abnormal serum ALT levels in a substantial proportion of individuals and the positive seroconversion were observed in only six volunteers who received higher doses of vaccine by the ELISA as late as 7.5 weeks after immunization.

DETAIL DESCRIPTION OF THE INVENTION

The virus strain of HAV was isolated from faces specimens derived from an hepatitis A patient in China. A 20% stool suspensions were prepared in PBS, and extract was prepared by differential centrifugation. The resultant extract was tested by conventional serological and morphological observation, and it demonstrates that some hepatitis A particles having size of about 27nm were presented therein. This stool extract was used as viral inoculum.

Rhesus monkey kidney culture cell monolayers were used for virus propagation. The cells were maintained in maintenance medium (Eagle's minimal essential medium, MEM) supplemented with 2% inactivated fetal bovine serum (FBS). The cells were allowed a 2-hour period of absorption, after which they were again sustained in maintenance medium and cultivated at35-36.degree. C. and the medium was changed at weekly intervals.By six weeks, the original maintenance medium was discarded, and the cells were harvested by

treatment with trypsin-EDTA and were disrupted by repeatedly freeze-thaw. The obtained virulent strain were attenuated by serial 15 passaging in the same host cells under monitoring by direct immunofluorescence (IE) for hepatitis A antigen at every passage. After additional 5 passages, the desired attenuated twentieth HAV H_2 (HAV H_2M20) was obtained.

Then, the resultant HAV H_2M20 virus were passaged five more times in human fetal lung fibroblast cells, the KMB17 cell line, at about 32-34 degree C., to provide passage 25. Experimental observation suggests that additional in vitro cultivation of the virus will yield a strain suitable for vaccine development. Obtained HAV H_2M20K5 (it was called H_2 virus strain for short) have been deposited at China Center for Type Culture Collection in Wuhan, China under CCTCC designation number 92001.

Compared to original virulent strain, so obtained attenuated strain H_2 evoked high titers of antibody response in HAV sensitive rhesus monkeys received the strain, and no abnormal elevation of aminotrasferase levels or cytopathic effect of liver cells were observed. Also, stools from all experimental animals received the vaccine were negative for hepatitis A virus immediately after immunization.

HAV strain H_2M20K5 were adapted an additional 2 passages in KMB17 cells to obtain the desired attenuated HAV live vaccine.

Antibody to hepatitis A was observed in all of the 139 health volunteers who received the vaccine by the intramuscular route for 6 weeks following the immunization. No local or systemic complaints were present immediately after immunization or during long-term follow-up, and serum alanine aminotransferase levels remained normal in all individuals during the period of 12 weeks observation. Furthermore, more than 95% of overall anti-HAV seroconversion rates were achieved after 3 weeks of vaccination. Selected sera tested for neutralizing antibody had titers ranging from 1:1 to 1:64 in these volunteers. Stools from 4 volunteers who received the vaccine were negative for hepatitis A virus during 8-30 days follow-up.

Claims

1, A method for preparing hepatitis A live vaccine strain, characterized by separating and adapting to 15 passages in newborn rhesus monkey kidney cell monolayer, and then further adapting 5 passages at a lower temperature of 32℃, wherein the HAV H_2 M20 strain have been deposited in CCTCC under accession number 92001.

2, A method according to claim 1, characterized by the strain was obtained by separating HAV from feces specimen of a hepatitis A patient, and then to adapting in vivo in the same newborn rhesus monkey kidney cell.

3, A method according to claim 1, characterized by the strain was attenuated at a

lower temperature of 32℃ and adapted to human lung diploid fibroblast cell KMB17.

4. A method according to claim 1, characterized by the animal models for immunogenicity and safety tests of the strain were HAV sensitive rhesus monkey.

Note: The text as above have completely and clearly described the substantial technical features of the invention, thus other sections of the specification in English are omitted.